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Improvement of *Rhizobium* Inoculants

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A practical approach was used to develop a *Rhizobium* (*Bradyrhizobium*) *japonicum* inoculant that increases soybean (*Glycine max* (L.) Merr.) yield in fields with indigenous *Rhizobium* populations, which typically outcompete strains present in existing commercial inoculants and therefore decrease the value of inoculant use. Field tests managed by several universities in the Mississippi delta region averaged a 169-kg/ha ($P < 0.01$) grain yield increase. The inoculant contains a mixture of mutants selected for increased nitrogen fixation ability. These mutants were derived from indigenous wild-type strains that are capable of high-level occupancy of nodules in soybean fields in the Mississippi delta region. To ensure microbiological purity, the inoculant is fermented directly in the point-of-use container with a vermiculite carrier (L. Graham-Weiss, M. L. Bennett, and A. S. Paau, Appl. Environ. Microbiol. 53:2138–2140, 1987). It should be possible to use this approach to produce more effective *Rhizobium* inoculants for any legume in any geographical area.

It has long been a challenge to researchers working with *Rhizobium* spp. to develop an inoculant that can promote higher levels of nitrogen fixation in legumes such as soybeans under practical field conditions. When other factors, such as moisture and temperature, are not limiting, higher levels of nitrogen fixation may improve grain yield.

Many commercial inoculants contain *Rhizobium* strains capable of high levels of nitrogen fixation. Although occasional yield increases have been reported in selected tests (7, 10), the use of these inoculants to improve soybean yield in major soybean-growing fields generally has not been successful (8, 12, 15, 19) because inoculant strains often compete unsuccessfully for nodule occupancy with the indigenous strains (15). In 1978, Maier and Brill (18) described mutants of *Rhizobium* (*Bradyrhizobium*) *japonicum* having increased nitrogen fixation ability. The mutants, however, were derived from a wild-type strain that was not competitive under most field conditions. Except when used in fields with no or very small populations of indigenous *Rhizobium* spp., the mutants failed to represent any significant portion of the total nodule *Rhizobium* population, fix more nitrogen for the host, or increase yield (21; Tom Wacek, Research Seeds, Inc., St. Joseph, Mo., unpublished results).

Many factors, including the particular *Rhizobium* strains (9, 26) and legume hosts (4, 14), soil type, moisture level, and temperature (17), play major roles in affecting the competitiveness of *Rhizobium* strains. Despite the advances in *Rhizobium* sp.-legume physiology and molecular biology research, few clues are available to improve inoculant products. Cho et al. (3) isolated mutants capable of more rapid nodulation of soybean seedlings. Competitiveness of these mutants under field conditions, however, remains to be determined. Components in root exudates, including various flavone and flavanone derivatives, have also been reported to increase nodule occupancy levels of some *Rhizobium* strains in greenhouse studies (1). Application to field conditions has not been reported.

In this article, we describe a simple strategy to develop an improved, practical *Rhizobium* inoculant for soybean. The strategy is based on the following assumptions. (i) Dominant, competitive indigenous strains are specific to a particular geographic region as a result of natural selection by both the local environment and the soybean varieties commonly used in the area. (ii) These indigenous strains, when isolated, added to the seed in high numbers (e.g., $>10^4$ bacteria per seed), and planted in the same geographic region, under favorable moisture and temperature conditions will establish themselves in the legume nodules. (iii) Mutants with increased nitrogen fixation ability can be isolated from these indigenous strains and remain competitive.

MATERIALS AND METHODS

Target geographical region and strain collection. Nodule samples were collected from soybean fields in southeast Missouri, west Tennessee, west Mississippi, and east Arkansas and from soybean fields in Louisiana along the Mississippi River. These areas are included in a region generally referred to as the Mississippi delta and represent a concentrated, major soybean-growing area (25). Soybean varieties cultivated in this region range from the late-maturing group IV to group VIII and are mostly of the indeterminate type. Soybean root nodules were sterilized by sequential submersion of the nodules in the following solutions for the specified amount of time, followed by a rinse with sterile distilled water for 5 min in between submersions: 2% Tween 80, 2 min; 95% ethanol, 5 min; 2% Clorox, 5 min; 3% stabilized hydrogen peroxide, 2 min. The nodules were finally rinsed with sterile distilled water three times for 1 min each time. The surface-sterilized nodules were then macerated in a yeast extract-mannitol (YEM) broth containing (per liter) 10 g of mannitol, 1 g of yeast extract, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.65 g of $K_2PO_4 \cdot 3H_2O$, 6.7 mg of $FeCl_3 \cdot 6H_2O$, and 4.2 μl of HCl. The nodule extracts were spread on YEM agar plates. A random portion of the colonies formed was cultured in YEM broth for 10 days with constant agitation at 26°C.

Strain characterization. Individual strains were grouped by the use of one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Kamicker and Brill (15). After the individual strains were grouped, strains within the same groups were reanalyzed in side-by-side comparisons. The estimated error rate of this method was determined to be no higher than 15%. Isolates were analyzed extensively in side-by-side comparisons with many members of the same groups in order to distinguish between closely related strains. The advantages and the reliability of this method in strain identification have been

TABLE 1. Relative abundance of two indigenous type strains (LA1304 and LA1325) in nodules collected from the Mississippi delta region

State	Abundance of strains in group[a]:				
	I	II	III	IV	V
Louisiana	LA1325	LA1304	B	B	B
Mississippi	LA1304	LA1325	A	A	C
Arkansas	LA1304	A	LA1325	A	A
Tennessee	A	LA1304	B	B	B
Missouri	LA1325	A	A	A	A

[a] Groups I to V represent indigenous strains in decreasing order of relative abundance. Nodules samples were collected during the 1982 growing season. Abbreviations: A, additional abundant type strains which were distinguishable from LA1304 and LA1325 (they are not identified by strain numbers in this report); B, additional strains which were present in nodules at a very low frequency (<5%) and not considered as abundant; C, no additional strains detected. Nodules from 15 to 20 plants were collected from four to six fields in the top two soybean-producing counties in each of the states. Isolates from three nodules of each plant were analyzed. Type strains LA1304 and LA1325 were not detected in nodules collected from the midwestern states Iowa, Illinois, Minnesota, Indiana, and Ohio.

documented (6, 13, 20, 22, 27, 28). More than 10,000 isolates were typed by using the method in this study.

Mutant selection and greenhouse evaluation. Selected isolates from soybean root nodules were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and screened on soybean seedlings by the method of Maier and Brill (18).

Field evaluation. From 1984 to 1987, various formulations containing specific mutants were field tested in multiple geographical locations either in replicated small-plot tests or in long-row strip tests, depending on the size of the field equipment available. When small-research-scale equipment was available, replicated small plot tests in a randomized complete-block design with at least 4 blocks were performed. When only farming size equipment was available, alternating strips (>0.2 ha per strip) of uninoculated control seedlings and treated seedlings were planted. Yield of the treated strip was then compared with the yield of the two adjacent control strips. The formulations included liquid, granular peat, sedge peat, and vermiculite. In 1987, one inoculant formulation (11) containing five mutants was selected and sent to state agricultural universities for independent evaluations. Field tests were performed by universities in the Mississippi delta region in the following states: Louisiana, 5 tests; Missouri, 1 test; and Tennessee, 2 tests. One of the tests in Tennessee was performed in the eastern part of the state and was not considered as part of the Mississippi delta region. Two tests were originally conducted in eastern Arkansas. These tests, however, were rejected because they were stressed severely by the weather and had a coefficient of variation of >17%. All replicated small-plot tests performed by the universities were either in a randomized complete block design or in a completely randomized design with at least four replications. Yield results were analyzed by a one-tailed *t* test with paired or nonpaired observations, depending on the test design. For multiple test comparisons, the significance level was estimated as the combined *P* value by the method of Sokal and Rohlf (23).

RESULTS AND DISCUSSION

The nodule occupancy results of indigenous strains isolated from the Mississippi delta region are summarized in Table 1. In Louisiana, indigenous strains belonging to two gel groups (represented by type strains LA1304 and LA1325) were dominant in 1982. Together, these two groups represented 60% of the *Rhizobium* population in the soybean nodules. Strain LA1304 also was the predominant strain in nodule samples collected from Mississippi and Arkansas and was the second most dominant strain in samples collected from Tennessee. Strain LA1325 was the most dominant one in Missouri, the second most dominant one in Mississippi, and the third most dominant one in Arkansas.

TABLE 2. Nitrogen fixation by mutants derived from the two competitive type strains, LA1304 and LA1325, on soybean seedlings

Mutant strain (no. of samples)	Parent strain (no. of samples)	% Increase in acetylene reduction[a]	Significance level[b]	Growth conditions[c]
T363 (77)	LA1325 (77)	29	0.00005	Greenhouse
T489 (85)	LA1325 (77)	20	0.0005	Greenhouse
T1344 (77)	LA1325 (76)	39	0.00005	Greenhouse
K567 (327)	LA1304 (317)	17	0.0001	Phytotron
K567 (101)	LA1304 (88)	54	0.0001	Greenhouse
S258 (388)	LA1304 (317)	13	0.001	Phytotron
S258 (104)	LA1304 (88)	67	0.0001	Greenhouse

[a] Nitrogen fixation activity was determined by the acetylene reduction assay on soybean seedlings by the method of Maier and Brill (18).

[b] The significance level was obtained by using a *t* test with nonpaired observations.

[c] Plants grown in the greenhouse received natural light and supplemental light. Plants grown in the phytotron received no natural light and were slightly etiolated.

An inoculant containing strains LA1304 and LA1325 was tested in one soybean field each in Louisiana and Iowa in 1983. Field tests were performed in Opelousas, La., and in Mt. Pleasant, Iowa. Both fields had previously been used to plant soybean and had indigenous *Rhizobium* strains. All the uninoculated control plants in 1983 nodulated as well as the inoculated plants did. The inoculants were prepared in sedge peat (24) and applied as seedhopper treatments to deliver 10^4 to 10^5 bacteria per seed. This concentration was equivalent to that of most commercial inoculants. In the Louisiana field, the inoculant increased nodule occupancy of the two strains by 30%. In Iowa, the inoculant failed to significantly increase the nodule occupancy rate of these same strains (total occupancy was 5%). These tests supported our belief that the dominant, competitive indigenous strains isolated from one geographical locale would remain competitive in that locale when used in an inoculant but might not be competitive when applied to a totally different geographical region. Two recent reports by Dowdle and Bohlool (5) and Klubek et al. (16) support this concept.

The two type strains, LA1304 and LA1325, representing the two most competitive gel groups in Louisiana, were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and screened on soybean seedlings (18). Mutants with a greater ability to reduce acetylene (a bioassay for nitrogen fixation) than that of their wild-type parents were collected at a frequency of approximately 10^{-3}. The activity of five of the mutants is summarized in Table 2. Under greenhouse or phytotron conditions, the mutants exhibited activities 13 to 67% higher than those of their parent strains.

From 1984 to 1987, various formulations containing the five mutants shown in Table 2 were tested under various field conditions. The field evaluation program was very similar to that described by Bradley et al. (2), with emphasis on the overall performance of the inoculants. Test results from one year provided the direction for improvement and optimiza-

TABLE 3. Yield results from independent field evaluations in the Mississippi delta region by state universities of a vermiculite-based *Rhizobium* inoculant containing mutants derived from the competitive wild-type strains

Location	kg/ha grain yield		Avg grain yield change		Significance level
	Control plants	Inoculated plants	kg/ha	%	
Louisiana					
1	2,520	2,692	172	6.8	0.21
2	2,149	2,253	104	4.8	0.20
3	2,559	2,951	392	15.3	<0.01
4	3,029	3,373	344	11.4	<0.01
5	2,343	2,382	39	1.7	0.36
Missouri	3,032	3,104	72	2.4	0.26
Tennessee	1,960	2,022	62	3.2	0.26
Avg	2,513	2,682	169	6.7	<0.004[a]

[a] Combined *P* value.

tion of the test inoculants with regard to (i) the choice of formulations, (ii) the geographic location of the test sites, (iii) the responses of the inoculant strains to soybean cultivars, and (iv) the use of the individual strains versus mixtures for the tests in the subsequent years. The 4-year program included 163 tests. In 1984, 29 small-plot tests were performed in nine different states with an average yield increase of only 0.7% ($P = 0.184$). In 1985, 12 small-plot tests and 3 long-row strip tests were performed in eight states with average yield increases of 3.6% ($P = 0.07$) and 5% ($P = 0.022$), respectively. In 1986, 97 long-row strip tests were performed in four southern states to test seven formulations containing a mixture of the five mutants shown in Table 2. The weighted average yield increase of the seven formulations was 6.3% ($P < 0.1$). One of the formulations performed very consistently and increased yield by 6.8% ($P < 0.01$). The formulation was a vermiculite-based inoculant and was tested again in 1987 along with one variation and two other formulations in 22 tests in two southern states. The weighted average yield increase of the two vermiculite-based inoculants was 5.2% ($P < 0.1$). In 1987, this vermiculite-based inoculant (11), containing the five mutants, was also sent to several state agricultural universities in the Mississippi delta region for evaluation in fields with indigenous *Rhizobium* populations. The vermiculite-based inoculant was chosen because it was microbiologically pure, it contained $>10^9$ rhizobia per g, it was capable of delivering $>10^5$ rhizobia per soybean seed, it had superior seed-coating properties, it was easy and inexpensive to produce, and it was amenable to large-scale production (11).

The performance of this inoculant in the different states is illustrated in Table 3. All the test sites had indigenous *Rhizobium* populations. All uninoculated plants were fully nodulated. As anticipated, the best inoculant performance was obtained from tests in Louisiana, where the wild-type parents from which the mutants had been derived were originally isolated. The average grain yield increase was 210 kg/ha, or 8% ($P < 0.01$). For tests that were in the Mississippi delta region, the original target for the use of this inoculant, a significant average yield increase of 169 kg/ha, or 6.7% ($P < 0.01$), was achieved.

These results demonstrate the feasibility of our strategy to develop a practical, effective *Rhizobium* inoculant targeted for the Mississippi delta region. There remain opportunities to further improve the performance of this inoculant in the delta region by developing and including additional mutants derived from some of the competitive indigenous strains representative of Tennessee, Arkansas, and Missouri (Table 1). By obtaining information on the competitive indigenous strains from other geographical regions, the same strategy can be used to develop a superior inoculant for these areas.

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